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Intracellular calcium concentration and activation of the Na^+/H^+ exchanger in essential hypertension

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Intracellular calcium concentration and activation of the Na^+/H^+ exchanger in essential hypertension. To investigate the relationship between changes in intracellular calcium concentration ($[Ca^{2+}]_i$) and agonist-induced activation of the Na^+/H^+ exchanger in essential hypertension (EH), platelet $[Ca^{2+}]_i$ and pH_i were measured in 24 patients with EH (14 males) aged 48 ± 2 years and 23 matched normotensive controls (NT) (12 males) aged 45 ± 3 years. Measurements were done with spectrofluorimetry using the dyes Fura-2 for $[Ca^{2+}]_i$ and BCECF for pH_i. $[Ca^{2+}]_i$ and pH_i were measured in the resting condition and after stimulation *in vitro* with 0.1 U/ml human thrombin. The thrombin-induced rise in pH_i was Na^+ dependent and amiloride sensitive, indicating that it was mediated by the Na^+/H^+ exchanger. Unstimulated $[Ca^{2+}]_i$ was higher in patients with EH than in NT (60 ± 3 vs. 48 ± 1 nmol/liter, $P < 0.005$), but there were no differences in resting pH_i between both groups (7.16 ± 0.01 vs. 7.16 ± 0.008). In the presence of 1 mmol/liter external calcium (Ca^{2+}_o), thrombin-induced increment in $[Ca^{2+}]_i$ was significantly greater in patients with EH than in NT (281 ± 21 vs. 206 ± 19; $P < 0.05$) as was the pH_i increment (EH: 0.137 ± 0.01; NT: 0.095 ± 0.01; $P < 0.05$). Both agonist-induced increments in $[Ca^{2+}]_i$ and in pH_i were correlated with mean arterial pressure (MAP) only in the EH group ($r = 0.58$, $P < 0.005$ and $r = 0.59$, $P < 0.005$, respectively). The agonist-induced rise in pH_i was positively correlated with the rise in $[Ca^{2+}]_i$ both in the EH group ($r = 0.65$, $P < 0.001$) and in the NT ($r = 0.55$, $P < 0.01$). At higher doses of thrombin (2.5 U/ml), the enhanced increment in both platelet $[Ca^{2+}]_i$ (EH: 732 ± 20; NT: 619 ± 15 nmol/liter, $P < 0.05$, $N = 6$) and in pH_i (EH: 0.193 ± 0.001; NT: 0.150 ± 0.004 $P < 0.005$, $N = 6$) was also observed in patients with EH. In the absence of Ca^{2+}_o (chelated with EGTA), there were no significant differences between patients with EH and NT in thrombin-induced increases neither in $[Ca^{2+}]_i$ (EH: 140 ± 18; NT: 121 ± 22 nmol/liter) nor in pH_i (EH: 0.098 ± 0.01; NT: 0.079 ± 0.01). When the agonist-induced increase in $[Ca^{2+}]_i$ was prevented with the Ca^{2+}_i chelator MAPTAM, no rise in pH_i was observed in either group. In conclusion, in platelets challenged with thrombin, the agonist-induced activation of the Na^+/H^+ exchanger occurs in correlation with the increment in $[Ca^{2+}]_i$, and both are significantly increased in patients with EH only in the presence of Ca^{2+}_o. Furthermore, the magnitude of the agonist-induced changes in $[Ca^{2+}]_i$ and in pH_i showed a direct relationship with MAP in the EH group. The agonist-induced activation of the Na^+/H^+ exchanger requires a rise in $[Ca^{2+}]_i$ both in patients with EH and in NT subjects.

A variety of cellular abnormalities in intracellular calcium concentration ($[Ca^{2+}]_i$) regulation have been described in a number of cell types in essential hypertension (EH) [1, 2]. Elevated concentration of $[Ca^{2+}]_i$ has been reported in platelets from subjects with EH both in the resting state [3–5] and after stimulation with thrombin [6]. Platelets have been used for analysis of intracellular calcium metabolism in EH because of the similarity of the calcium-dependent contractile processes between these cells and vascular smooth muscle cells (VSMC) [7]. In this regard, both cell types are activated by a rise in $[Ca^{2+}]_i$ which is manifested by smooth muscle cell contraction and platelet aggregation. Therefore, platelet elevated $[Ca^{2+}]_i$ in EH may reflect increased VSMC reactivity which would be expressed as increased vascular resistance and elevated blood pressure.

Recently, it has been demonstrated that intracellular pH (pH_i) plays an important role as an intracellular signal for extracellular hormonal messages [8, 9]. In this regard, the changes in pH_i have been found to produce marked effects on contraction in smooth muscle [9, 10]. Abnormalities in resting pH_i have been described in circulating cells from essential hypertensives as compared to normotensives with variable results: increase [11, 12], no difference [13, 14] or decrease in pH_i [15]. It is known that pH_i is regulated in part by the membrane Na^+/H^+ exchanger which exchanges intracellular H^+ for extracellular Na^+ [8–10, 16]. The Na^+/H^+ exchanger is known to be responsive to vasoconstrictors, growth factors, intracellular acidification and cell shrinking [8–10, 16, 17]. More consistently, Na^+/H^+ exchanger activity in response to intracellular acidification has been reported to be elevated in EH [11, 14, 18–20]. The importance of the Na^+/H^+ exchanger activity in EH is suggested by: (1) the broad number of contractile functions potentially regulated by pH_i [10]; (2) its critical involvement in cell growth and proliferation [16], which may explain the abnormal hypertrophy and proliferation of VSMC from hypertensive patients and spontaneously hypertensive rats (SHR) with respect to controls; and (3) its importance as a source of Na^+ reabsorption in the epithelium of the renal proximal tubules [21].

The changes observed in $[Ca^{2+}]_i$ and pH_i in patients with EH suggest that there may be a mutual control of these parameters which could be important in the pathophysiology of EH. However, there is controversy with respect to the interrelationship between agonist-induced rise in $[Ca^{2+}]_i$ and activation of the Na^+/H^+ exchanger [16]. The purpose of this study was to evaluate the relationship between agonist-induced rise in platelet $[Ca^{2+}]_i$ and activation of the Na^+/H^+ exchanger in EH in an

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Table 1. Clinical and biochemical characteristics of the subjects studied

	Hypertensives	Normotensives
Number (males)	24 (14)	23 (12)
Age *years*	48 ± 2	45 ± 3
Systolic BP *mm Hg*	173 ± 4[a]	109 ± 2
Diastolic BP	108 ± 1[a]	71 ± 1
Mean BP	129 ± 1[a]	83 ± 1
BMI kg/m^2	27 ± 0.7[b]	25 ± 0.6
PRA *ng/ml · hr*	0.43 ± 0.08	0.34 ± 0.05
NE *pg/ml*	274 ± 21	263 ± 28

Abbreviations are: BP, blood pressure; BMI, body mass index; PRA, plasma renin activity; NE, norepinephrine. Values are means ± SEM.
[a] $P < 0.001$
[b] $P < 0.05$

attempt to further our knowledge about the role of $[Ca^{2+}]_i$ in the agonist-mediated activation of the Na^+/H^+ exchanger.

Methods

Materials

Human thrombin from Sigma (St. Louis, Missouri, USA) was resuspended in distilled water and stored at −20°C until use. Fura-2-acetoxymethylester (Fura-2-AM), 2',7'-bis (carboxyethyl)-5,6-carboxyfluorescein tetraacetoxymethylester (BCECF-AM) and MAPTAM (1,2-bis-5-methyl-amino-phenoxylethane-N,N,N'-tetra-acetoxymethyl acetate) were from Calbiochem (La Jolla, California, USA), prepared in dimethyl sulfoxide (DMSO) and stored at −20°C until use. EIPA [3-amino-5-ethylisopropilamino-6-chloro-N-(diaminoethylene) pyrazine-carboxamine hydrochloride] was provided by Merck Sharp & Dohme, Spain. Nigericin, EGTA, digitonin, choline chloride and N-methyl-D-glucamine were from Sigma. All other chemicals were of analysis grade purchased from Merck (Darmstadt, Germany).

Patients

Twenty-four Caucasian patients with EH and twenty-three Caucasian normotensive subjects were included in the study after informed consent. Subject clinical and biochemical characteristics are shown in Table 1. None of the normotensive control subjects had a family history of hypertension. Patients were free of antihypertensive medication for at least three weeks prior to the study, and none of the subjects were receiving medications known to affect platelet function at least 10 days prior to the study. Subjects were excluded for the following: secondary causes of hypertension, diabetes mellitus, abnormal renal function, congestive heart failure, alcoholism and women on birth control pills. Subjects were studied in the fasting state between 8:00 and 10:00 a.m. An indwelling cannula was inserted in an antecubital vein, and after a 60-minute supine rest blood pressure was recorded with a conventional sphygmomanometer. The actual blood pressure used was the average of three determinations separated five minutes. Fifty ml of venous blood were sampled without stasis for measurement of $[Ca^{2+}]_i$ and pH_i. Mean arterial blood pressure was calculated as the diastolic pressure plus one third the pulse pressure. Body mass index (BMI) was calculated as weight (kg)/height $(m)^2$.

Biochemical determinations

Preparation of cells. Fifty ml of venous blood was drawn into 20% (vol:vol) acid-citrate-glucose (2.5 g sodium citrate, 1.5 g citric acid, 2.0 g glucose in 100 ml distilled water) and was immediately centrifuged at 200 × g for 10 minutes at 20°C to obtain the platelet-rich plasma (PRP). EGTA 0.5 mmol/liter was added to prevent platelet activation and the PRP was centrifuged at 500 × g for 20 minutes at 20°C. The pellet was resuspended in HEPES-buffered saline (HBS) consisting of (in mmol/liter) NaCl 145, KCl 5, $MgCl_2$ 1, glucose 6, HEPES 10 and bovine serum albumin (BSA) 0.2 mg/ml, pH 7.4 at 37°C. The platelet suspension was incubated either with 2 μmol/liter Fura-2-AM or 0.75 μmol/liter BCECF-AM for 35 minutes at 37°C in a shaking water bath protected from light. The labeled platelets were passed through a 2.5 × 10 cm Sepharose CL2B column (Pharmacia, Uppsala, Sweden) preequilibrated with HBS to remove the extracellular dye. Platelet count was adjusted to 0.5×10^8/ml using a Technikon H-1 System coulter counter (Technikon Instruments Corp., Tarrytown, New York, USA). $CaCl_2$ to 1 mmol/liter was added to replenish intracellular calcium stores. After an equilibration time of 15 minutes at 37°C, 2 ml of the platelet suspension were placed in a 3 ml quartz cuvette for fluorescence measurements.

Fluorescence measurements

Fluorescence measurements were carried out in a Hitachi F-2000 spectrofluorometer (Hitachi, Ltd. Tokyo, Japan). The sample was contained in a 3 ml quartz cuvette placed in a cuvette chamber thermostated at 37°C. During the individual fluorescence readings the platelet suspension was continually stirred with a magnetic stirrer at low speed to prevent platelet activation. All additions were made directly into the cuvette through a small hole in the top of the cuvette chamber with microsample syringes. The fluorescence signal was obtained once every second with alternate excitation wavelengths at 340 and 380 nm for Fura-2 and 500 and 440 nm for BCECF. $[Ca^{2+}]_i$ and pH_i were estimated by the ratio of the excitation wavelengths 340/380 and 500/440, respectively. The emission wavelength was 510 nm for Fura-2 and 530 nm for BCECF. Autofluorescence of the unlabeled cell suspension with the vehicle DMSO was recorded before and after lysis with digitonin and subtracted from the total fluorescence, and was always <15% and <6% of the fluorescence signal for Fura-2 and BCECF readings, respectively.

Calculation of $[Ca^{2+}]_i$. The relationship between fluorescence ratio at 340/380 and $[Ca^{2+}]_i$ was obtained using the equation

$$[Ca^{2+}]_i = K_d\,(R - R_{min})/(R_{max} - R) \times F_{min}/F_{max}$$

[22], where R_{max} is the ratio of fluorescence at saturating Ca^{2+}, R_{min} is the ratio at zero Ca^{2+}, F_{min} and F_{max} are the fluorescences at 380 nm in zero Ca^{2+} and in saturating Ca^{2+}, respectively. K_d is the effective dissociation constant of Fura-2 in appropriate conditions and was taken to be 224 nmol/liter, according to Grynkiewicz, Poenie and Tsien [22]. Calibration was performed by the following procedure: after each single experiment, cells were lysed with digitonin 50 μmol/liter in the presence of 1 mmol/liter $CaCl_2$ to obtain R_{max} and F_{max}. Thereafter, to obtain R_{min} and F_{min}, EGTA 6 mmol/liter was

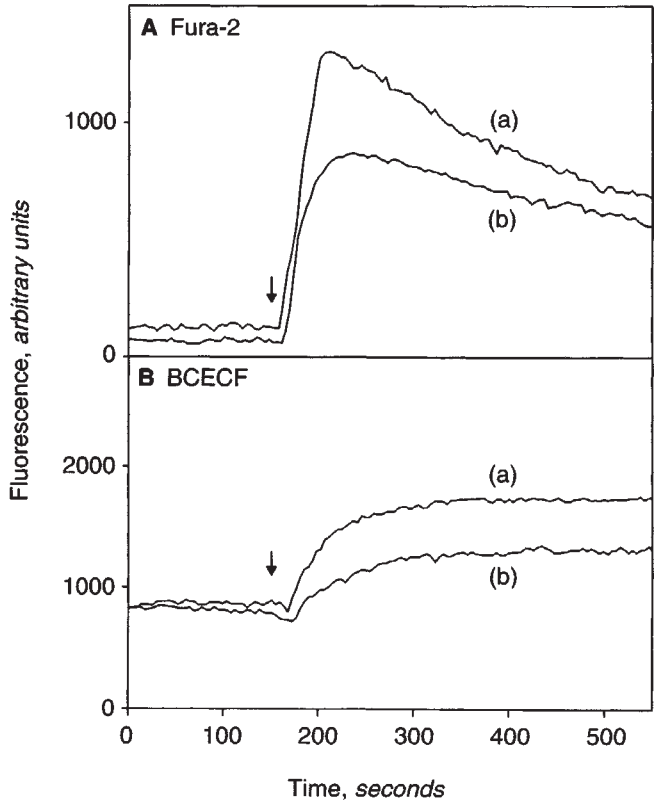

Fig. 1. *Representative traces of (A) Fura-2 fluorescence at 340 nm and (B) BCECF fluorescence at 500 nm before and after stimulation with 0.1 U/ml human thrombin (arrow) of platelets of a hypertensive patient (a) and a normotensive control (b).*

added and the pH of the cuvette was adjusted to 8.3 with 20 mmol/liter Tris base.

$[Ca^{2+}]_i$ was measured under resting conditions, that is, with unstimulated platelets and after stimulation with 0.1 U/ml thrombin. The increment of $[Ca^{2+}]_i$ in nmol/liter ($\Delta[Ca^{2+}]_i$) was calculated by subtracting baseline $[Ca^{2+}]_i$ from the maximal response to thrombin (Fig. 1).

Calculation of pH_i. The fluorescence signal of BCECF was calibrated to pH at the end of every single experiment. Platelets were lysed with digitonin 50 μmol/liter and the fluorescence signal was recorded at known values of pH from 6.5 to 8 simultaneously monitored by a combined microelectrode (Hamilton, Bonaduz AG, Switzerland) inserted directly in the cuvette. The pH_i estimated from this calibration was corrected for the shift in the excitation maximum of intracellular and extracellular BCECF according to the method of Thomas et al [23]. Platelets were resuspended in high K^+ HEPES buffer which contained in mmol/liter: KCl 120, NaCl 20, glucose 6, $MgCl_2$ 1, HEPES 10 (pH 6 at 37°C) and nigericin 2 μg/ml allowing the external pH to equal pH_i. The external pH was increased stepwise from 6 to 8 and the fluorescence signal was recorded. The cells were then lysed with digitonin and the pH decreased stepwise back to pH 6. The difference between the intracellular and the postlysis calibrations curves showed that the signal in the latter underestimated pH_i by 0.13 ± 0.02 pH units (mean ± SEM; $N = 21$). The values presented in this work have been corrected according to these data.

Intracellular pH was measured under resting conditions and after stimulation with 0.1 U/ml of human thrombin. The increment of pH_i in pH units was calculated by subtracting the baseline pH_i from the response at 300 seconds after stimulation with thrombin (Fig. 1).

Manipulation of the Na^+/H^+ exchanger

As indicated above, experiments were performed in a nominally free bicarbonate buffer to assess the role of the Na^+/H^+ exchanger. Two methods were employed for inhibition of the Na^+/H^+ exchanger. First, the sodium of the HBS was isoosmotically replaced by choline chloride. Secondly, the amiloride-derivative specific exchanger-blocker, EIPA 50 μmol/liter, was used [24]. Understanding the kinetics of the Na^+/H^+ exchanger under our experimental conditions, the activity of the exchanger was measured by acidifying BCECF-loaded platelets (1 μmol/liter) with nigericin 0.5 μg/ml in sodium free HBS without BSA (Na replaced isosmotically by N-methyl-D-glucamine) of different pH values (at least 7 values ranging from 6 to 7.2) essentially as described by Gardner et al [25]. After 10 minutes with nigericin the Na^+/H^+ exchanger was activated by adding 100 μl aliquots of the pre-acidified platelets (2×10^8/ml) to the cuvette containing 2 ml of sodium-HBS plus BSA (pH 7.4 at 37°C). The dilution of the nigericin plus the BSA eliminated any further effect of the ionophore and allowed the pH_i recovery. This recovery was completely inhibited by 50 μmol/liter EIPA, indicating that it was mediated by the Na^+/H^+ exchanger. The rate constants of every single pH_i-dependent recovery curve from acidification were obtained through nonlinear regression analysis of the data following the equation:

$$pH_{i,t} = pH_{i,\infty} - [pH_{i,\infty} - pH_{i,0}] \times 1^{-kxt}$$

where $pH_{i,t}$ is pH_i at a given time t, $pH_{i,\infty}$ is pH_i at the new steady state, $pH_{i,0}$ is pH_i at the initial time t = 0 (at the beginning of pH_i recovery), and k is the rate constant. This equation was also assumed for the earliest part of the curve, when recording was disturbed by mixing artifacts [25]. The apparent initial rate of pH_i recovery was calculated as the derivative at the initial point. The relation between initial pH_i and the initial rate of pH_i recovery was fitted to a sigmoidal model [26], and the maximal initial velocity (V_{max}) was computed as the top plateau of the curve. The pH_i value for half maximal activation of the exchanger ($pH_{i0.5}$) was also obtained. To examine the kinetics of sodium activation of the Na^+/H^+ exchanger, the initial rate of pH_i recovery was evaluated at different extracellular sodium concentrations (10, 40, 70, 100 and 140 mmol/liter) at a $pH_{i,o}$ of 6.4. The relation between extracellular sodium and the initial rate of pH_i recovery was fitted to a sigmoidal model to obtain the Km for external sodium. All mathematical calculations were obtained using the computer program GraphPAD-INPLOT4 (GraphPAD Software, San Diego, California, USA).

Intracellular Ca^{2+} (Ca^{2+}_i) clamp

Five minutes after addition of BCECF, 40 μmol/liter of the intracellular calcium chelator MAPTAM were added to the loading medium and incubated for 30 minutes at 37°C. Afterwards, cells were washed as described above. Before addition

Table 2. $[Ca^{2+}]_i$ and pH_i measurements in hypertensives and normotensives

	Hypertensives	Normotensives
$[Ca^{2+}]_i$ *nmol/liter*	60 ± 3[a]	48 ± 1
pH_i (pH units)	7.16 ± 0.01	7.16 ± 0.008
$\Delta[Ca^{2+}]_i$ Ca_o	281 ± 21[b]	206 ± 19
ΔpH_i Ca_o	0.137 ± 0.01[b]	0.095 ± 0.01
$\Delta[Ca^{2+}]_i$ EGTA	140 ± 18	121 ± 22
ΔpH_i EGTA	0.098 ± 0.01	0.079 ± 0.01
pH_i MAPTAM	6.95 ± 0.02	6.90 ± 0.02
ΔpH_i MAPTAM	−0.06 ± 0.008	−0.05 ± 0.008

The thrombin-induced increases in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$; nmol/liter) and pH_i (ΔpH_i; pH units) were calculated by subtracting the baseline from the maximal response and from the response at 300 seconds, respectively. Abbreviations are: Ca_o, presence of external calcium 1 mmol/liter; EGTA, blockade of external calcium entry to the cell with EGTA 1.2 mmol/liter; MAPTAM, blockade of $[Ca^{2+}]_i$ increase with the intracellular calcium chelator MAPTAM 40 μmol/liter. Values are means ± SEM.

[a] $P < 0.005$

[b] $P < 0.05$

of the agonist, EGTA 1.2 mmol/liter was added to the platelet suspension.

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Statistical comparisons were determined by Student's test for unpaired data. Correlation coefficients were calculated by the least squares method. Results were similar using non-parametric tests. A value of $P < 0.05$ was considered statistically significant.

Results

Clinical and biochemical characteristics of the subjects are summarized in Table 1. There were no differences with respect to age, sex and the hormonal profile between both groups. Under unstimulated conditions, platelet $[Ca^{2+}]_i$ was significantly higher in patients with EH than in NT (60 ± 3 vs. 48 ± 1 nmol/liter, $P < 0.005$; Table 2). However, there were no differences in basal pH_i between both groups (EH: 7.16 ± 0.01, NT: 7.16 ± 0.008 pH units). The agonist-induced rise in $[Ca^{2+}]_i$ and the activation of the Na^+/H^+ exchanger was evaluated by stimulation of the platelets with 0.1 U/ml of thrombin in the presence of 1 mmol/liter external calcium ($Ca^{2+}{}_o$), in the absence of $Ca^{2+}{}_o$, and with the intracellular calcium chelator MAPTAM. In the presence of 1 mmol/liter $Ca^{2+}{}_o$, thrombin-induced rise in $[Ca^{2+}]_i$ was significantly greater in patients with EH than in the NT controls (281 ± 21 vs. 206 ± 19, $P < 0.05$; Table 2; Fig. 2). Under these circumstances, thrombin-induced rise in pH_i was also significantly greater in the EH patients (0.137 ± 0.01 vs. 0.095 ± 0.01, $P < 0.05$) as compared to the NT. The increment in pH_i reflects activation of the Na^+/H^+ exchanger as it was abolished when platelets were pretreated with EIPA 50 μmol/liter, a specific inhibitor of the exchanger, or resuspended in HBS in which sodium was isosmotically substituted by choline chloride (data not shown). To know about the kinetic parameters of the Na^+/H^+ exchanger under our experimental conditions, we evaluated in a group of patients and controls ($N = 6$) the maximal initial velocity (V_{max}) (EH: 0.195 ± 0.005, NT: 0.123 ± 0.001 $dpH_i/30$ s, $P = 0.078$),

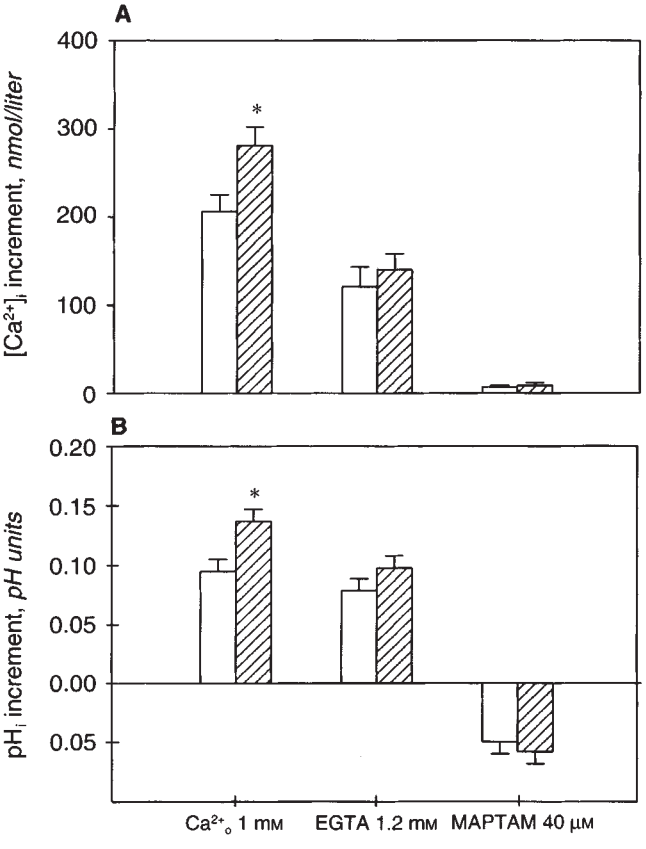

Fig. 2. *Thrombin-induced increments in $[Ca^{2+}]_i$ (nmol/liter) (A) and in pH_i (pH units) (B) in hypertensives (▨) and normotensives (□): (1) in the presence of 1 mmol/liter external calcium ($Ca^{2+}{}_o$); (2) in the absence of extracellular calcium, chelated with EGTA 1.2 mmol/liter prior to the addition of thrombin; and (3) in cells loaded with the intracellular calcium chelator MAPTAM 40 μmol/liter).* Bars represent means ± SEM, * $P < 0.05$.

$pH_{i0.5}$ (EH: 6.60 ± 0.03, NT: 6.65 ± 0.007, P = NS) and the Km for extracellular sodium (EH: 63.2 ± 3, NT: 60.6 ± 2, P = NS).

At higher doses of thrombin (2.5 U/ml), the enhanced increment in both platelet $[Ca^{2+}]_i$ (EH: 732 ± 20; NT: 619 ± 15 nmol/liter, $P < 0.05$, $N = 6$) and in pH_i (EH: 0.193 ± 0.001; NT: 0.150 ± 0.004; $P < 0.005$, $N = 6$) was also observed in patients with EH. Both the thrombin-induced increases in $[Ca^{2+}]_i$ and in pH_i were positively correlated with MAP in the EH group (for $[Ca^{2+}]_i$ $r = 0.58$, $P < 0.005$; for pH_i $r = 0.59$; $P < 0.005$; Fig. 3). It is important to note that the thrombin-induced rise in pH_i was positively correlated with the increment in $[Ca^{2+}]_i$ in both the EH group ($r = 0.65$; $P < 0.001$) and in the NT controls ($r = 0.55$; $P < 0.01$; Fig. 4). Nevertheless, unstimulated $[Ca^{2+}]_i$ and pH_i were not correlated and each showed no significant correlations with age or blood pressure in either group when considered separately.

The thrombin-induced rise in $[Ca^{2+}]_i$ as well as in pH_i was also evaluated in the absence of extracellular calcium ($Ca^{2+}{}_o$) by chelation with EGTA 1.2 mmol/liter prior to the addition of the agonist. Under this condition, the rise in $[Ca^{2+}]_i$ represents mobilization of calcium from intracellular stores. In contrast to the results obtained in the presence of 1 mmol/liter of $Ca^{2+}{}_o$, in the absence of this ion no differences were observed in the thrombin-induced increase of $[Ca^{2+}]_i$ between the two groups

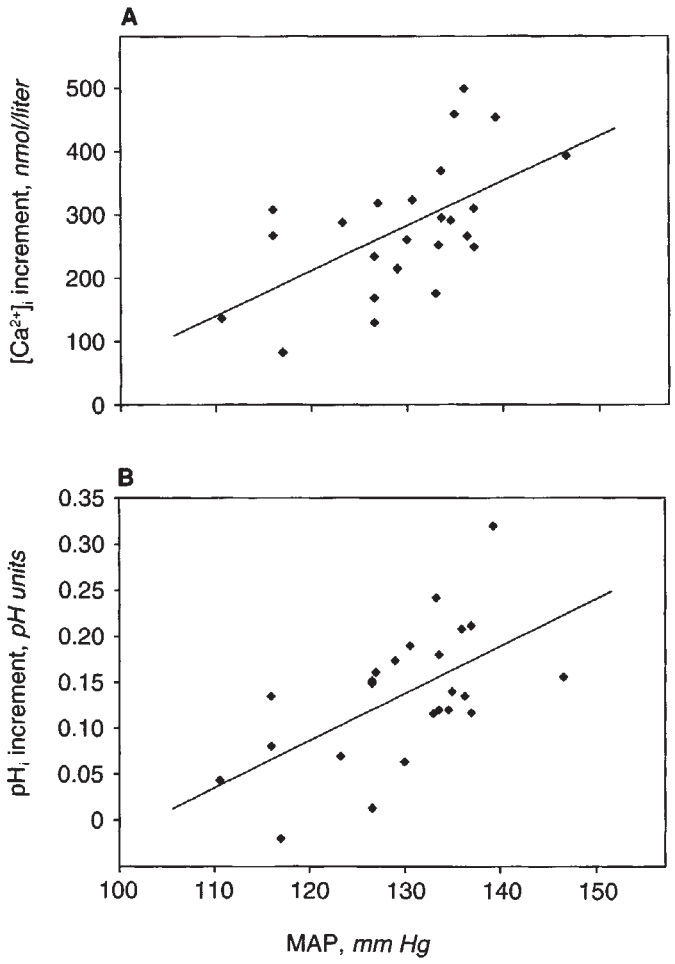

Fig. 3. *Relationship between mean arterial pressure and thrombin-induced increment in* (**A**) $[Ca^{2+}]_i$ *(nmol/liter;* r = 0.58; P < 0.005*) and* (**B**) *in* pH_i *(pH units;* r = 0.59; P < 0.005*) in the hypertensive group.*

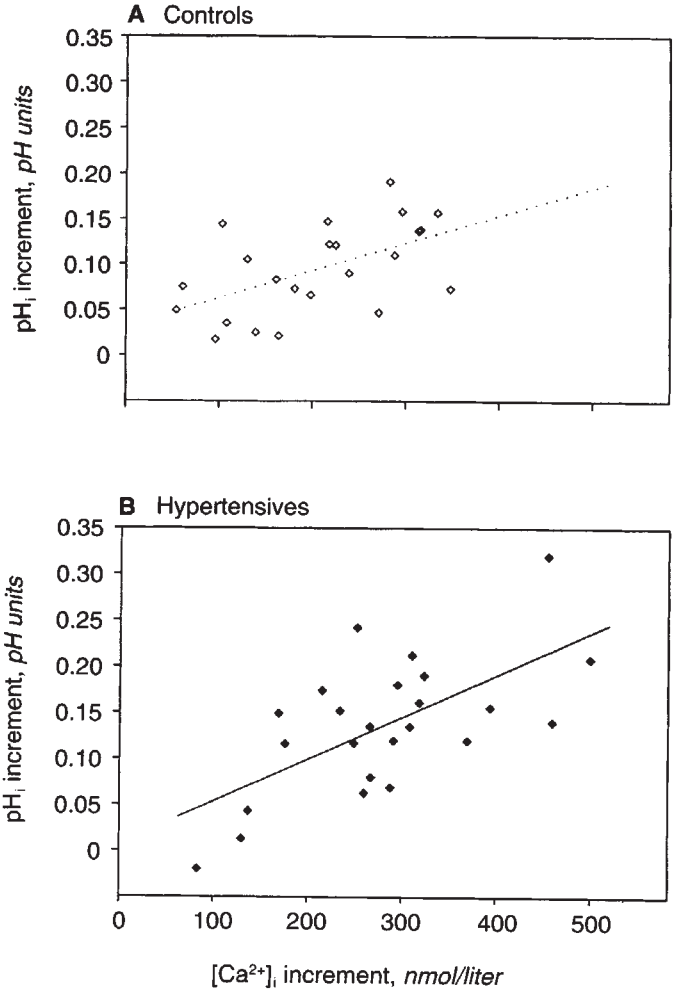

Fig. 4. *Relationship between thrombin-induced increments in* $[Ca^{2+}]_i$ *(nmol/liter) and in* pH_i *(pH units) in* (**A**) *controls* (r = 0.55; P < 0.01*) and* (**B**) *hypertensives* (r = 0.65; P < 0.001*).*

(EH: 140 ± 18, NT: 121 ± 22, $P = 0.27$; Table 2; Fig. 2). As well, the previously observed difference in the thrombin-induced increase in pH_i between both groups was no longer present in $Ca^{2+}{}_o$ free medium (EH: 0.098 ± 0.01, NT: 0.079 ± 0.01, $P = 0.25$; Table 2).

When $Ca^{2+}{}_i$ response to thrombin was abolished with preincubation of platelets with the intracellular calcium chelator MAPTAM, no significant increase in $[Ca^{2+}]_i$ was observed (data not shown) (Fig. 2). Under this condition, basal unstimulated pH_i was more acidic than in calcium repleted conditions in both groups (EH: 7.16 ± 0.01 vs. 6.95 ± 0.02, $P < 0.001$ and NT: 7.16 ± 0.008 vs. 6.90 ± 0.01, $P < 0.001$). With thrombin, no elevation in pH_i was observed. On the contrary, a slight intracellular acidification was observed in both groups (EH: −0.06 ± 0.008; NT: −0.05 ± 0.008, P = NS; Fig. 2).

Discussion

In the present investigation, we have measured both $[Ca^{2+}]_i$ and pH_i in patients with EH and in NT in the resting state and after stimulation *in vitro* with human thrombin. It is demonstrated that stimulation of platelets produced increases in $[Ca^{2+}]_i$ and in pH_i, which were significantly greater in patients with EH than in NT controls. The agonist-induced increment in pH_i showed a close relationship with the rise in $[Ca^{2+}]_i$ in both groups of subjects suggesting a central role of $Ca^{2+}{}_i$. In this regard, in calcium-depleted platelets, thrombin was unable to induce activation of the Na^+/H^+ exchanger in either group.

Disturbances in $Ca^{2+}{}_i$ metabolism have been reported in experimental and in human hypertension in a variety of cell models [1, 2]. Unstimulated $[Ca^{2+}]_i$ in VSMC has been reported to be higher in SHR than in normotensive, Wistar-Kyoto rats (WKY) [27]. Furthermore, arginine-vasopressin-induced increases in rat VSMC $[Ca^{2+}]_i$ is higher in SHR as compared to WKY [28]. In human EH an increased basal platelet $[Ca^{2+}]_i$ is a widely established finding [3–6]. In addition, platelets of essential hypertensives exhibit a greater degree of agonist-induced increase in $[Ca^{2+}]_i$ [6], reflecting platelet hyperresponsiveness. In our hands, the increased response in agonist-induced rise of $[Ca^{2+}]_i$ in essential hypertensives was due to an increase in calcium entry into the cell, since no difference in cellular calcium mobilization (in the absence of extracellular calcium) was found between EH and NT. This data are in agreement with ^{45}Ca influx studies performed in rat VSMC [29] and with studies performed in human platelets [6], suggesting an

abnormality in the cell membrane transport of Ca^{2+} in EH. The parallelism of the alterations in the metabolism of $Ca^{2+}{}_i$ between platelets and VSMC is supported by our finding of a significant correlation between agonist-induced increase in $[Ca^{2+}]_i$ and MAP in the EH group only in the presence of extracellular calcium. In this regard, in experimental hypertension the calcium antagonist drugs and not other hypotensives produce greater hypotensive effects than in control animals, suggesting that enhanced vasoconstriction is maintained by membrane calcium channel activity [2].

A number of cell types display an increase in pH_i upon stimulation with agonists and growth factors, and this alkalinization is mediated by stimulation of the amiloride-sensitive Na^+/H^+ exchanger [8, 9, 16]. Recently, this exchanger has been cloned [30], and four isoforms have been identified (NHE-1–NHE-4) [31]. NHE-1 is amiloride sensitive and seems to be expressed in all cell types with basolateral localization in polarized cells [31]. NHE-2 has reduced susceptibility to inhibition by amiloride and seems to be expressed in polarized cells, in an apical localization [31]. NHE-3 and NHE-4 are localized in intestine and stomach, respectively, and their functional properties are currently being investigated. The Na^+/H^+ exchanger activity in response to intracellular acidification has been reported to be elevated in circulating cells of patients with EH both in the absence [11, 18–20] and in the presence of bicarbonate in the medium [14]. The amiloride sensitivity of the Na^+/H^+ exchanger activity determined in most of these studies argues against the participation of the NHE-2, and at present, it is not known whether NHE-3 and NHE-4 are expressed in blood cells. The method employed in studies evaluating the activity of the Na^+/H^+ exchanger requires the measurement of the initial rate of pH_i recovery from an acid load, and in this condition transport activity is assessed at pH_i values below physiologic resting pH_i. We also measured the kinetics of the Na^+/H^+ exchanger in response to intracellular acidification in a group of patients and controls. Although the differences between the small groups of patients and controls were not statistically significant, the tendency displayed was in agreement with the majority of reports. The activation of the Na^+/H^+ exchanger has also been studied in response to agonists in rat VSMC [32, 33] and has been found to be significantly increased in the SHR with respect to WKY rats only in early subcultures of VSMC [34]. To our knowledge at present, increased agonist-induced rise in pH_i has not been reported in human hypertension. Our study, performed in the nominal absence of bicarbonate, demonstrates a significantly greater agonist-induced intracellular alkalinization, reflecting activation of the Na^+/H^+ exchanger, in platelets of patients with EH as compared to NT controls, despite no differences found in resting pH_i between both groups. As occurred with agonist-induced increments in $[Ca^{2+}]_i$, agonist-induced increases in platelet pH_i were also significantly correlated with MAP in the EH group. Both the enhanced rises in platelet $[Ca^{2+}]_i$ and pH_i seen in patients with EH were observed at low and high doses of thrombin, suggesting an increased responsiveness of platelets from patients with EH.

The concomitant increases in $[Ca^{2+}]_i$ and in pH_i upon agonist activation suggest an interrelationship between these two parameters. Nevertheless, there is controversy about the relationship between $[Ca^{2+}]_i$ elevations and cytoplasmic alkalinization. Some authors have reported that activation of the Na^+/H^+ exchanger after agonist stimuli is a prerequisite for Ca^{2+} mobilization in human platelets [35]. It has been stated that in EH, the mechanisms involving an increase in intracellular Na^+ ($Na^+{}_i$) are responsible of the elevation of $[Ca^{2+}]_i$ through the membrane Na^+-Ca^{2+} exchange [36]. The increase in $Na^+{}_i$ is thought to be mediated by a circulating inhibitor of the Na^+-K^+-ATPase [36, 37]. Otherwise, activation of the amiloride-sensitive Na^+/H^+ exchanger also represents a source of $Na^+{}_i$ which would be capable of generating increases in $[Ca^{2+}]_i$ [35]. Nevertheless, it has been recently demonstrated that activation of the Na^+/H^+ exchanger by different stimuli is unable to produce any measurable effect on $[Ca^{2+}]_i$ in rat VSMC [38]. Likewise, the total platelet $[Ca^{2+}]_i$ seems to be independent from the transmembrane Na^+ gradient [39]. Conversely, several reports have pointed out that agonist-induced $Ca^{2+}{}_i$ mobilization stimulates the Na^+/H^+ exchanger in human fibroblasts [40] and in rat VSMC [41]. Moreover, $[Ca^{2+}]_i$ elevation might be a necessary step for the activation of the exchanger [41–43]. In addition, non-agonist-induced increases in $[Ca^{2+}]_i$ are capable of stimulating the Na^+/H^+ exchanger in a variety of cell models [40, 42]. The biochemical mechanisms responsible for the calcium induced activation of the Na^+/H^+ exchanger may involve both protein kinase C-dependent and -independent pathways [33, 44], the latter possibly mediated by calcium calmodulin processes [43, 45]. In this respect, it is noteworthy that protein kinase C activity has been shown to be elevated in platelets of the SHR [46].

As hypothesized by Aviv [47], it is conceivable that the association between elevated $[Ca^{2+}]_i$ and hyperactivity of the Na^+/H^+ exchanger could be a unifying explanation for the increased vasoactive response, the proliferative process of vascular muscle cells, and salt sensitivity, which are hallmarks of EH. The results presented here agree with a Ca^{2+} requirement for activation of the Na^+/H^+ exchanger since in calcium-depleted platelets, thrombin was unable to activate the exchanger. The agonist-induced intracellular alkalinization showed a close relationship with the agonist-induced increase in $[Ca^{2+}]_i$ in both groups of subjects. In agreement with a hyperresponsive state, patients with EH showed significantly greater agonist-induced increases in $[Ca^{2+}]_i$ and in pH_i as compared to the NT subjects. The results of the present work are in concordance with the hypothesis that a hyperactive Na^+/H^+ exchanger in EH could be secondary to an abnormality in the metabolism of $Ca^{2+}{}_i$ [47]. The source of the increased basal and agonist-induced increase in $[Ca^{2+}]_i$ has not been firmly established [48], but our data suggest a disturbance in the membrane transport of Ca^{2+}, since both the hyperresponse in stimulated $[Ca^{2+}]_i$ and pH_i in EH were abolished in the absence of extracellular Ca^{2+}.

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